

Bioprocess for semi-industrial production of immunomodulator polysaccharide Pleuran by *Pleurotus ostreatus* in submerged culture

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Pleuran, is a polysaccharide belongs to glucan group which made of D-glucose moieties linked with (1→3)-β and (1→6)-β glycosidic linkages. The importance of this compound is based on its wide biotherapeutic application as immunomodulator and anticancer polysaccharide. This compound is produced only naturally using specific type of mushroom named *Pleurotus ostreatus*. Traditionally, this type of mushroom was cultivated using solid state cultivation system in green houses. Nowadays, submerged cultivation is considered as alternative cultivation strategy for mushroom based on its many advantages such as: short cultivation time, high yield, fully controlled cultivation condition, and fewer steps in polysaccharide extraction and purification compared to solid state fermentation. Thus, the present work was focused on the development of pleuran production process in semi-industrial scale using submerged cultivation system. At first, high yield production medium was selected followed by study on the kinetics of cell growth and EPS production in shake flasks. Second, cultivations were conducted in semi-industrial scale using *in situ* sterilizable 16-L stirred tank bioreactor under controlled and uncontrolled pH conditions. The results showed that bioreactor cultivation under controlled pH condition improved EPS production process and the maximal volumetric and specific pleuran produced in this study were 1.98 g/L and 0.445 g/g, respectively.

Key words: Pleuran, β-D glucan, *Pleurotus ostreatus*, submerged culture, stirred tank bioreactor

Introduction

Pleuran (CAS # 159940-37-1) is one of the most important glucose polymers and belongs to (1,3/1,6)-D-glucan group. Like other hexose polymers, it has the general chemical formula of $(C_6H_{10}O_5)_n$. The importance of this compound is based on its potential activity as immunomodulator properties. This type of biopolymer is naturally produced by specific type of mushroom named *Pleurotus ostreatus*¹. Since centuries, mushrooms have been widely used as food in different cultures and reported their effect potentiality as healthy food and to increase the body defense and boost the immune system against many diseases². More recently, this polysaccharide was isolated and identified by different research groups. Hot water extraction of fruit body is the most common method for pleuran production process and the extracted product was used by several authors

as traditional medicine for cancer treatment³. The research on the chemical structure of this polysaccharide investigated numerous parameters including the molecular weight, degree of substitution, degree of branching, chain conformation in solution, sugar component and the structures of main and branches^{4,5}. The immunomodulator and anticancer properties of this polysaccharide was also verified using different types of cancer cells. It induced anti-proliferative and pro-apoptotic effect on colon cancer cells⁶. It possesses also strong growth inhibitory effect up to 95% on Sarcoma 180 cancer cells using mice model in *in vitro* study⁷. Moreover, pleuran showed also anti lipoperoxidative effect, antiviral, anti-oxidant and keratinocyte stimulatory effects in different animal models⁷⁻¹⁰. More recently, beside the common biotherapeutic properties of pleuran, this polysaccharide showed also potential application in nutraceutical industries based on its high prebiotic properties¹¹. Traditionally, this mushroom is cultivated in green house under controlled temperature and humidity.

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This method has many disadvantages such as long term cultivation up to several months, low yield of bioactive polysaccharide, long extraction and purification process. Moreover, as open cultivation system, the mushrooms could be easily contaminated by other microbes. As this process is running under fully uncontrolled cultivation conditions, the process is not complying with cGMP requirements and thus limited the application of the obtained polysaccharides for biopharmaceutical applications. Thus, cultivation under strict controlled cultivation conditions in submerged culture was matter of interest for many researchers^{5, 12}.

In our previous research, *P. ostreatus* was successfully cultivated in submerged culture and the produced polysaccharide was fully identified using IR and ¹³C NMR. Like the naturally produced polymer in mushroom fruit body, pleuran produced in submerged culture system showed also special pattern of highly branched chain of peptidoglycan with 1→3 and 1→6 linkages¹³. The isolated polysaccharide from submerged culture possesses also immunostimulatory effect and anticancer activity almost equal to the biopolymer obtained from mushroom fruit body. The objective of this work was to study the kinetics of cell growth and pleuran production in submerged culture system. First, media previously used in literature were used to select the most appropriate medium supporting high specific pleuran production. Second, kinetics of cell growth and EPS production were studied in both shake flask and bioreactor levels under different cultivation conditions to maximize both of volumetric and specific pleuran production yields.

Experimental Section

Microorganism

The strain used throughout this work was *Pleurotus ostreatus* NRRL B-366. This basidiomycetes fungal strain was kindly provided by ARS culture collection (Peoria, IL, USA). After receiving of strain, it was maintained and subcultured monthly on petri dish surface culture using sterile solid potato dextrose agar medium (PDA, Oxoid, UK). After inoculation, cells were incubated at 26 °C for 14 days and stored thereafter in a refrigerator at 5 °C for further use.

Inoculum preparation

Mushroom cells, obtained from previously stored agar plates, were initially grown on PDA medium in a Petri dish. After 14 days at 26°C, the sporulated surface

mycelia growth was harvested in sterile saline solution (9 g/L NaCl), and used to inoculate vegetative medium for inoculums broth preparation. The harvested cells were used to inoculate 2 L Schott bottle of 750 mL working volume equipped with one magnet for stirring and containing YM medium composed of (g/L): glucose, 10.0; yeast extract, 3.0; malt extract, 3.0; peptone, 5.0. The pH of medium was adjusted to 5.5 before sterilization. Fifty ml of homogenized mycelium, collected from petri dishes, were used to inoculate the 2-L schott bottle and the inoculated bottles were incubated for 7 days at 26°C on non-heat emission magnet stirrer (SI-300X, Scientific Industries Inc., NY, USA) adjusted at 110 rpm. After incubation, the grown cells were harvested, homogenized using Omni homogenizer (Omni International, USA) to prevent large aggregate formation and used to inoculate EPS production medium.

Media screening for EPS production

Six different media were used in this study for primary evaluation and selection of the best medium which supports high EPS production. All these media were reported before by different research groups for their ability to support mushroom cell growth and EPS production. The compositions of these media were as follows in (g/L): Medium (1): glucose, 40; Corn Steep Liquor, 20¹²; Medium (2): Malt extract, 250 ml; peptone, 5.0; K₂HPO₄, 2.0¹⁴; Medium (3): glucose, 20.0; peptone, 2.0; yeast extract, 2.0; KH₂PO₄, 0.46; K₂HPO₄, 1.0 and MgSO₄.7H₂O, 0.5¹⁵; Medium (4): glucose, 30.0; yeast extract, 20.0; KH₂PO₄, 3.0; MgSO₄.7H₂O, 3.0¹⁶; Medium (5): glucose, 20.0; soybean meal, 10.0; KH₂PO₄, 3.0; MgSO₄.7H₂O, 0.2¹⁷; Medium (6): Malt extract, 10.0; peptone, 1.0; potato dextrose broth, 24.0¹⁸; Medium (7): glucose, 10.0; malt extract, 3.0; peptone, 5; yeast extract, 3.0¹⁸. The pH of all media was adjusted to 5.5 before sterilization. The carbon sources were sterilized separately and added to the corresponding fermentation medium before inoculation. The inoculated flasks were incubated on the rotary shaker (Innova 4080, New Brunswick Scientific Co., NJ, USA) at 200 rpm and 26°C.

Medium for cell growth and EPS production

Cultivations were conducted in shake flask and bioreactor level. The selected production medium, which yielded the highest EPS production according to the medium screening experiment, was composed of (g/L): glucose, 20.0; peptone, 2.0; yeast extract, 2.0; KH₂PO₄,

0.46; K_2HPO_4 , 1.0 and $MgSO_4 \cdot 7H_2O$, 0.5. Glucose was sterilized separately and added to the cultivation medium before inoculation. The pH was adjusted to 5.5 before sterilization.

Shake flask and Bioreactor cultivations

For shake flask experiments, 250 ml Erlenmeyer flasks of 50 ml working volume were used. The flask were inoculated with homogenized mycelium as described previously in concentration of 5% (v/v) and incubated for 20 days at 26°C on a rotary shaker incubator (Innova 4080, New Brunswick Scientific, NJ, USA) at 200 rpm. Samples in form of two flasks each were collected every day and used for cell dry weight, EPS and glucose analysis.

Bioreactor cultivations

Cultivation in stirred tank bioreactor were conducted using the optimized medium in shake flask level and run under the same cultivation conditions in term of inoculums size, temperature and pH. The bioreactor used in this study was 16-L stirred tank bioreactor (BioEngineering, Wald, Switzerland) with working volume of 8-L. The stirrer was equipped with two 6-blade Rushton turbine impellers (d_i (impeller diameter) = 85 mm; d_t (tank diameter) = 214 mm, d_i/d_t = 0.397). The agitation speed was adjust to 200 rpm and kept constant throughout the cultivation and aeration was performed using filtered sterile air and supplied continuously to the bioreactor with rate of 1 v/v/min. Foam was suppressed by the addition of antifoam agent Struktul (Schill+Seilacher Gruppe, Hamburg, Germany). During the cultivation process, pH value and dissolved oxygen concentration were determined using pH and DO polarographic electrodes, respectively (Ingold, Mittler-Toledo, Switzerland). In case of pH controlled culture, the pH was adjusted to 5.5 by cascading the pH controller with acid/base feeding peristaltic pumps connected with 4 M HCl and 4 M NaOH solutions.

Analysis

Sample preparation and cell dry weight determination

Samples, in form of two flasks containing 50 ml each, or 25 ml of broth in case of bioreactor cultures, were taken at different times during the cultivation and collected in a centrifugation falcon tube (Falcon, USA). Immediately after sampling, cells were separated from the supernatant using cooling centrifuge at 5°C and 5000 rpm for 20 min. The supernatant was frozen immediately at -20°C and stored for EPS and glucose analysis. For cell dry weight determination, the pellets were washed twice by distilled

water, centrifuged again and dried in an oven at 80°C for constant weight.

Determination of total carbohydrates

Total carbohydrates analysis in culture was determined according to the method of Brooks *et al.*¹⁹. In this method, freshly prepared modified anthrone reagent, prepared by dissolving 0.1% (w/v) anthrone in diluted sulphuric acid (2.3:1 v/v ratio acid to water), was added to 25 µl sample and heated in screw capped test tube for 5 min in boiling water bath. The sample was removed and cooled in ice bath to stop the reaction. The absorbance of the developed color was measure at 630 nm using double beam spectrophotometer against blank (water blank of 25 µl treated in similar way).

Determination of glucose

Glucose was determined in culture broth using DNS method developed by Miller²⁰. In this method, 50µl sample was added to 600µl DNS solution (prepared by dissolving 10 g 3,5- dinitro salicylic acid; 2 g phenol, 0.9 sodium sulphate and 10.0 g sodium hydroxide in 1 L water) and 350µl citrate buffer solution (pH 6.5). The mixture was incubated for 5 min in shaking water bath at 95°C. The reaction was terminated by cooling in ice bath for 3 min. The absorbance of the developed color was determined against blank (50µl water treated in similar way) at 540 nm using spectrophotometer (DR/2500, Hach, CO, USA).

Determination of exopolysaccharides (EPS)

The frozen samples were thawed at first at room temperature. The polysaccharides in sample was obtained by mixing with cold ethanol in ratio (1:3; sample to ethanol) and kept for 20 hours at 4 °C to facilitate the precipitation process of EPS. The precipitated polysaccharides were centrifuged at 10.000 g for 20 min for full EPS separation. After discarding the supernatant, the precipitate of pure EPS was washed separately with ethanol, acetone and ethyl ether followed by lyophilization step²¹. After complete lyophilization cycle (3-4 days), the obtained EPS powder was weighed.

Results and discussion

Screening media for EPS production

This study was started with the investigation of medium composition effect on the cell growth and EPS production by *P. ostreatus*. Thus, seven different media of diversified composition as described in the materials and methods part were selected from previously published literatures

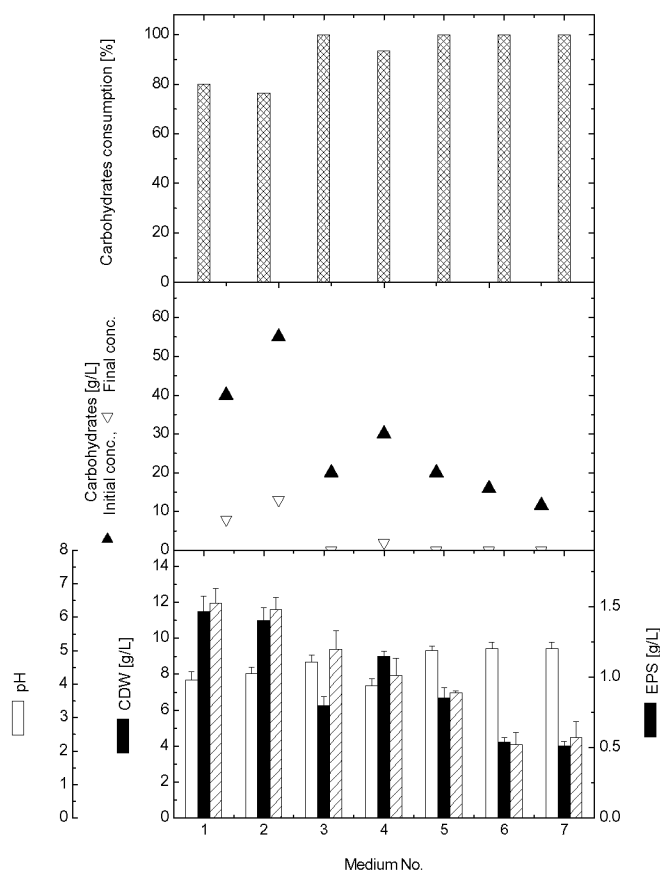


Fig. 1 Cell growth, EPS production and final pH after cultivation of *Pleurotus ostreatus* in different media, bars indicate standard deviation from triplicate determinations (data were taken after 20 days cultivation).

to investigate their potency for EPS production. Parallel cultivations were conducted in shake flask culture and the inoculated flasks were incubated at 26°C and 200 rpm. The results in figure 1 demonstrate the results of cell growth, EPS production and carbohydrate consumption after 20 days cultivation. As shown, the maximal cell dry weight of about 11.5 g/L was obtained in medium 1 which includes only glucose and corn steep liquor followed by medium 2 which composed of malt extract, peptone and phosphate salt. These two media yielded also the maximal volumetric EPS production of about 1.52 and 1.48 g/L, respectively. However, *P. ostreatus* grew in all media and produced EPS in different extent ranged between 1.20 g/L (medium 3) and 0.57 g/L (medium 7). In these media, different types of carbon sources were used and thus total carbohydrate analysis was performed for all culture. However, the carbon source in culture was consumed in different extend dependent on its initial concentration. For media of initial carbohydrate concentration less than 30 g/L, no

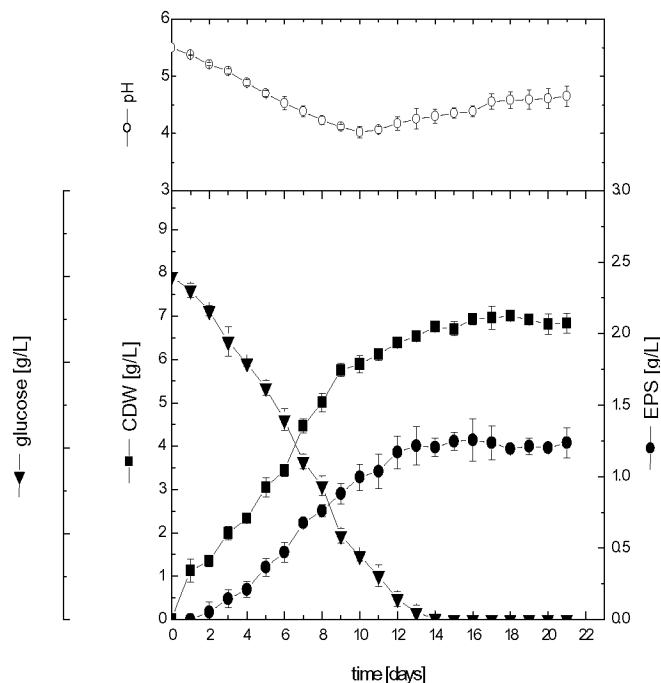


Fig. 2 Changes in the profiles of Cell growth, EPS production and pH during cultivations of *P. ostreatus* in shake flask culture (bars indicate standard deviation from triplicate determinations)

carbon source was left after 20 days cultivation. Whereas, in media 1 and 2 (of initial carbohydrate concentration of 40 g/L and 55 g/L, respectively) about 20% of the initial carbon source remain unconsumed after 20 days cultivation. To get better understanding about the suitability of the media under study for EPS production, different yield coefficients were also calculated. However, the maximal values of both of $Y_{[P/X]}$ and $Y_{[P/S]}$ of about 0.2 [g/g] and 0.06 [g/g] was obtained in medium No. 3. This directly indicate that, even though the medium 3 was the third best medium in terms of volumetric EPS production, it was ranked as the first for its ability to support highest EPS production yield. This indicates that when *P. ostreatus* grew in this medium, the grown cells give the highest performance for biopolymer production concomitant with the optimal use of carbon source for EPS production. The top two media for volumetric production were almost 30% less in specific production value compared to medium 3. Thus, this medium was selected for further study. The superiority of this medium may be due to its balanced nutrient composition and its enrichment with necessary complex nitrogen source such as yeast extract and peptone in combination with phosphate sources in balanced concentration in addition to necessary inorganic nutrients such as magnesium sulphate.

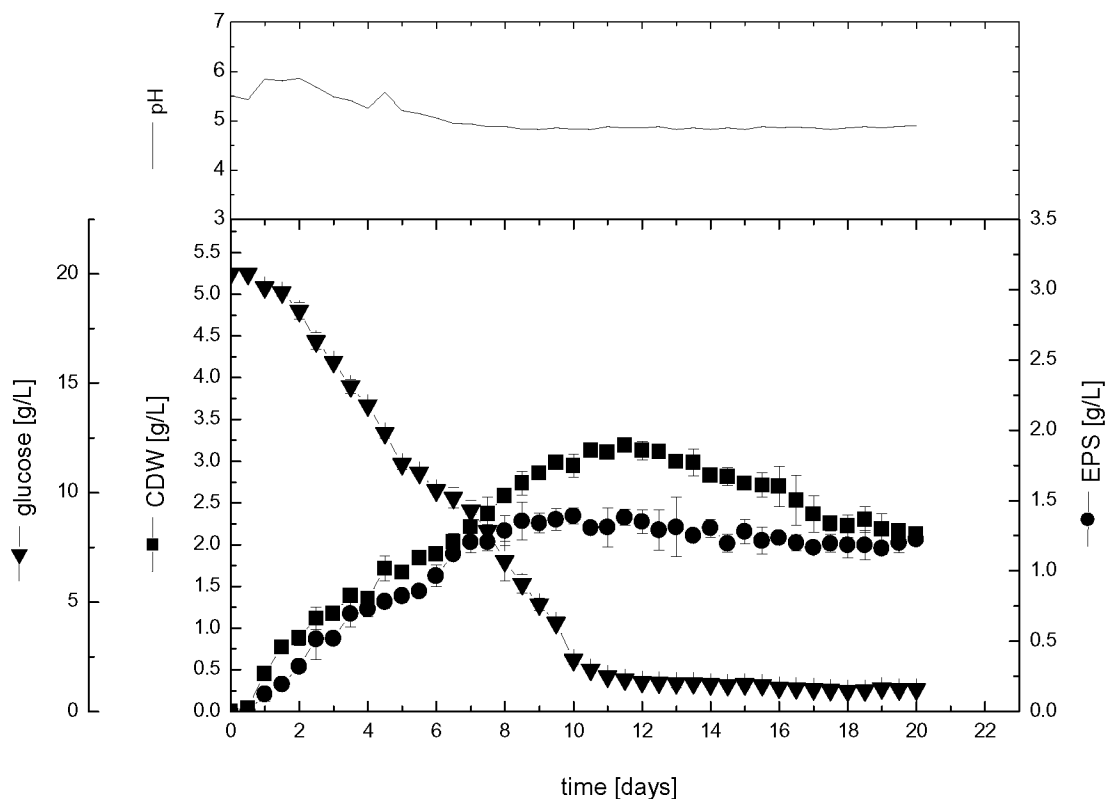


Fig. 3 Production of EPS by *P. ostreatus* in 16-L stirred tank bioreactor under un-controlled pH condition (bars indicate standard deviation from triplicate determinations)

Kinetics of cell growth and EPS production in shake flask

The aim of this experiment was to study the growth and EPS production kinetics in small scale cultivation. Figure 2 demonstrates the mushroom growth profile, substrate consumption and EPS production during cell cultivation in 250 mL Erlenmeyer flask. As shown, cells grew exponentially after inoculation with growth rate of 0.027 g/L/h reaching about 5.8 g/L after 9 days. Cells grew thereafter with lower growth rate and reached about 7.0 g/L after 16 days and kept more or less constant for the rest of cultivation time. During this active growth phase, glucose consumed with constant rate of about 0.064 g/L/h and reached almost zero after 14 days. For EPS production, after a lag phase of 2 days, the biopolymer produced in culture and accumulated with rate of about 0.0039 g/L/h in culture reaching its maximal value of 1.26 g/L after 13 days and kept more or less constant for the rest of cultivation time. This result is in agreement with other authors who also observed that this biopolymer is not consumed by its producer organism¹². Contrary to this finding, other researchers reported on EPS reduction in culture based on degradation/utilization by mushroom strains²². However, the process of EPS utilization in culture is may be dependent on type strain, molecular weight of the EPS produced and nutrient limitation in

culture. Based on these data it could be concluded that both of cell growth and EPS production were terminated as a result of carbon source (glucose) limitation in culture. The maximal volumetric EPS produced in this cultivation was almost like those obtained by other authors. On the other hand, the maximal specific EPS production [$Y_{P/X}$] was higher than those obtained in other studies. However, in shake flask culture, aeration/oxygen availability control in culture as well as pH control could be not achieved in this closed cultivation system. Thus, to get better understanding on the effect of these two process parameters, cultivations were conducted in stirred tank bioreactor.

Kinetics of cell growth and EPS production in semi-industrial scale 16-L stirred tank bioreactor using controlled and un-controlled pH conditions

In general, mushrooms are aerobic microorganisms and thus need continuous supply of oxygen for growth and different metabolites production. In this experiment, cultivations were conducted in 16-L *in situ* sterilizable stirred tank bioreactor. Medium was sterilized in bioreactor at 121°C for 30 min. During sterilization process, medium was agitated at 50 rpm to maximize the sterilize efficiency and to provide rapid heating/cooling

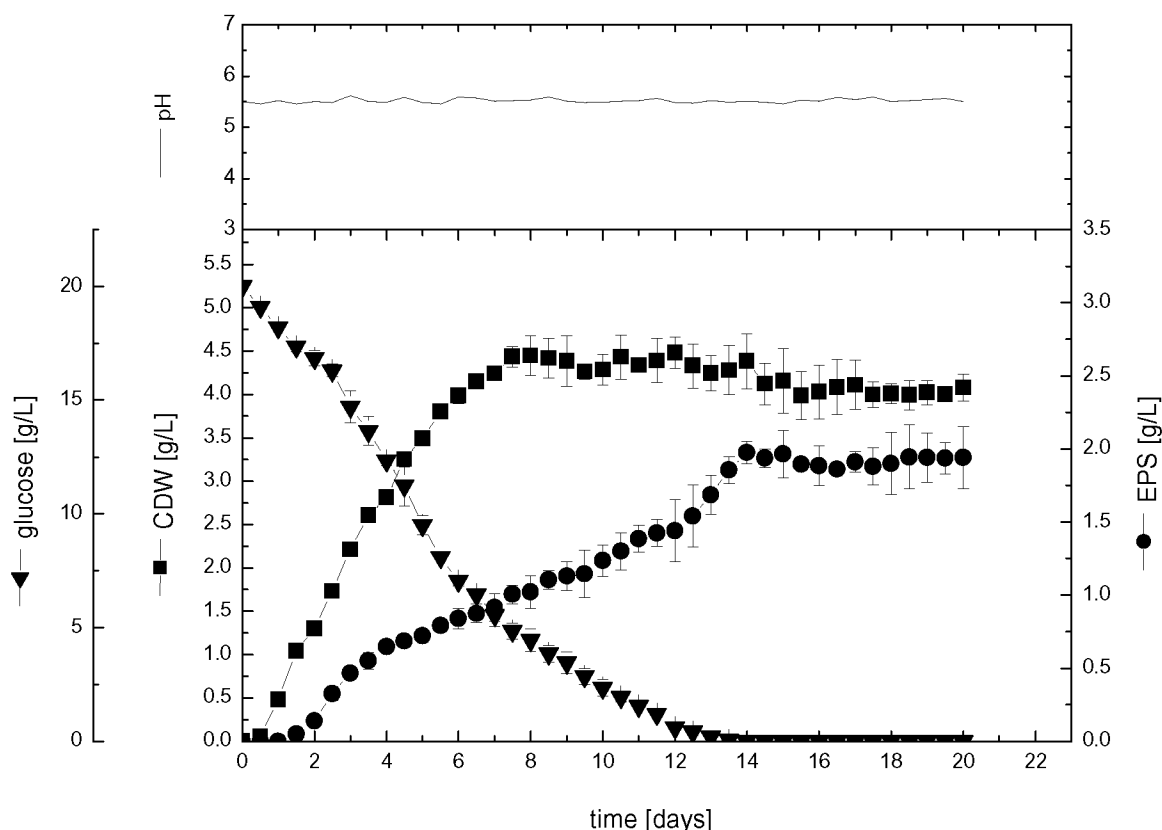


Fig. 4 Production of EPS by *P. ostreatus* in 16-L stirred tank bioreactor under controlled pH condition (bars indicate standard deviation from triplicate determinations)

of the bioreactor to minimize nutrient degradation process. Glucose was sterilized separately and added to the bioreactor medium before inoculation. Medium composition, inoculum size and cultivation conditions were the same as in shake flask experiment. Two parallel sets of experiments were performed using stirred tank bioreactor with 8-L working volume under controlled (pH 5.5) and uncontrolled pH conditions. The results of batch cultivation under uncontrolled pH condition are shown in figure 3. Cells grew exponentially after inoculation without significant lag phase and reached about 3.2 g/L after 11 days cultivation. During this phase, the cell growth rate and specific growth rate were, 0.013 g/L/h and 0.0088 h⁻¹, respectively. After that time cells entered short stationary phase and the biomass decreased gradually thereafter reaching about 2.5 g/L after 20 days. The decreased in biomass in this experiment compared to shake flask one may be attributed to the higher shear in bioreactor culture. Moreover, the growth morphology in this culture was of smaller pellet in mycelia network. This make also cells more sensitive to shear stress compared to the growth of cells in shake flask which was in form of compact pellet structure. In parallel to

cell growth, pleuran was also produced in culture during this active growth phase in rate of about 0.0069 g/L/h reaching maximal concentration of 1.2 g/L (almost the same obtained in shake flask culture). On the other hand, the specific EPS production was almost double of those obtained in shake flask. This directly indicates that the cell performance in bioreactor culture was higher than those in smaller scale shake flask. This may be attributed to cell morphology where cells in the first case was in form of small pellet in mycelia network whereas cells was in form of larger pellet in shake flask culture. This relation between morphology and productivity of filamentous microorganisms was reported by many authors²³⁻²⁵. The smaller the biopellet the higher oxygen and mass transfers of nutrient/product in the microbial system and this increase the rate of metabolite(s) production process^{26,27}.

In parallel to this experiment, cultivation was conducted in stirred tank bioreactor under controlled pH. In this experiment, the pH was kept constant at 5.5 by continuous addition of H₂SO₄/NaOH using computerized pH control system. As shown in figure 4, cells grew exponentially during the first 8 days with growth rate of

Table 1 Kinetics of cell growth and EPS production during batch cultivation in shake flasks and in stirred tank bioreactor under different cultivation conditions.

| Parameters | Shake flask | Bioreactor | |
|--------------------------|-------------|-----------------|---------------|
| | | pH uncontrolled | pH controlled |
| Growth parameters | | | |
| X_{\max} [g/L] | 7.00 | 3.20 | 4.45 |
| dx/dt [g/L/h] | 0.027 | 0.013 | 0.026 |
| μ [h ⁻¹] | 0.0094 | 0.0088 | 0.0119 |
| Production parameters | | | |
| P_{\max} [g/L] | 1.26 | 1.20 | 1.98 |
| Q_p [g/L/h] | 0.00389 | 0.00693 | 0.00639 |
| Q_s [g/L/h] | 0.064 | 0.070 | 0.057 |
| Yield Coefficients | | | |
| $Y_{p/X}$ [g/g] | 0.182 | 0.375 | 0.445 |
| $Y_{p/S}$ [g/g] | 0.063 | 0.063 | 0.099 |
| $Y_{X/S}$ [g/g] | 0.327 | 0.168 | 0.222 |

X_{\max} : maximal cell dry weight, dx/dt : growth rate, μ : Specific growth rate, P_{\max} : maximal EPS production; Q_p : EPS production rate; Q_s : glucose consumption rate, $Y_{p/X}$: specific EPS production (g EPS produced per g cell dry weight). $Y_{p/X}$: Yield of EPS on biomass; $Y_{p/S}$: Yield of EPS on glucose consumed; $Y_{X/S}$: Yield of cell on glucose consumed.

0.026 g/L/h. This value was almost double of those obtained in bioreactor culture without pH control. After that time, cells reached its maximal growth of about 4.45 g/L and were fluctuated thereafter between 4.3 to 4.0 g/L for the rest of cultivation time. During growth phase, glucose was consumed in culture with rate of 0.057 g/L/h. On the other hand, EPS was produced in culture after lag phase of 2 days and increased gradually with rate of 0.00639 g/L/h reaching its maximal concentration of 1.98 g/L after 14 days. This amount of EPS produced was almost 65% higher than those obtained in corresponding batch of the same cultivation conditions with exception of pH control. However, not only the volumetric EPS production increased in this culture but also the specific production [$Y_{p/X}$] in terms of [g EPS produced/ g cell dry weight] was increased as well by about 20%. This we can conclude that the increase in EPS production in pH controlled culture was a result of combination between higher cell mass production and increased cell production capacity as well.

Conclusion

Table 1 summarizes the kinetics of cell growth, glucose consumption and EPS production by *P. ostreatus* when cultivated in shake flask and in bioreactor under different cultivation conditions. It observed significantly that cultivation in bioreactor yielded lower cell mass with higher volumetric and specific EPS production. This is directly related to better mixing and aeration in bioreactor compared to shake flask. Moreover, cells grew in shake

flask in large pellet structure which is less preferred for fungal metabolite production. Cells cultivated in bioreactor under controlled pH condition yielded the highest volumetric and specific EPS production of 1.98 g/L and 0.445 g/g, respectively. The process developed in this study shows high potential for industrial scale production of the EPS pleuran in submerged culture with significant reduction of batch production time to only 14 days compared to more than 3 months cultivation in conventional solid state culture.

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